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14. ABSTRACT

We are studying the utility of using *B.fragilis* OmpA as a vehicle on which to put antigenic epitopes of organisms that can be used in bioterror, with the aim of eventually constructing a vaccine vehicle vector. OmpA is the major outer membrane protein of *B. fragilis*, a gram negative anaerobe that normally resides in the gut. There are four homologs for *ompA* in the genome. The purpose of this study was to construct a *B. fragilis ompA* deletant and to begin to characterize the function of OmpA, as well as the specific function(s) of the various loops exposed on the surface. We constructed an *ompA1* deletant (WAL 186) and confirmed the deletion by sequence analysis of the deletion junction. RT PCR indicated that all 4 *ompA*s are transcribed in the parental strain, and confirmed that *ompA1* is not transcribed in WAL 186 (the *ompA1* deletant). However, we found that *ompA4* was also not transcribed in the deletion mutant. No significant change was seen in MICs of a variety of antimicrobials for the deletion mutant compared to the parental strain. WAL 186 was more sensitive than WAL 108 to high salt and to SDS. Gram stain analysis showed no change was seen between WAL 108 and WAL 186 grown overnight in normal media (long, somewhat pleomorphic rods), but overnight growth in hyperosmolar media (200 mM NaCl) resulted in very small, round forms for both WAL 108 and WAL 186. In addition, expression of *ompA1* and *ompA4* was diminished in WAL 108 (the parental strain.). We will use this OmpA deletant todetermining specific loop functions and design a vaccine.

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Table of Contents

Introduction

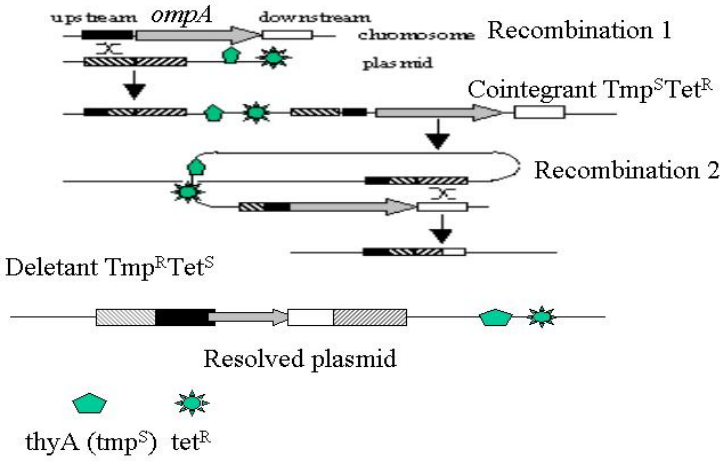
The dangers of bioterror bacterial or viral agents have been of concern to the military and political leaders for decades. Methods to prevent infection with these agents and to treat infections that may occur are being studied with intensity. Historically, vaccines have been the most efficient method of handling diseases in large populations. Studies of the immune system and vaccine effectiveness have shown that the ideal way to induce a complete immune response of both the mucosal and systemic systems is to administer vaccines in a manner that mimics the natural route of infections. Over the past 15 years, experimental bacterial vaccine vectors have been produced that elicit immune responses against bacterial, viral, protozoan and metazoan pathogens in laboratory animals. Among the advantages of these vaccines is that they are relatively inexpensive to manufacture, they can be given orally, and they can be treated with antibiotics if desired and they effectively induce both humoral and cellular responses. If the organism used as the vector can potentially colonize in the host, the potential of eliciting the appropriate response is increased.

OmpA proteins are among the most conserved of all outer membrane proteins in bacteria; however, the loops expressed on the outer surface are quite variable. Current understanding indicates that the loops are responsible for a variety of virulence characteristics and that they serve as important antigens as well. *Bacteroides fragilis*, most common anaerobic pathogen, is a major component of the stool flora and colonizes the gastrointestinal tract. OmpA is one of the major outer membrane proteins of this organism. Structure/function analysis of the OmpA protein, which will ultimately identify the exposed loops of the protein will both elucidate the role of OmpA in the pathogenic process of *Bacteroides fragilis* and will allow us to exploit the unique nature of this abundant outer membrane protein in designing potential vaccine vectors. These vaccines (i.e., *Bacteroides fragilis* with OmpA modified to express specific epitopes) could be designed for a wide variety of infections that might be acquired by ingestion of food or liquid (including potential biodefense related organisms), and the secretory IgA antibodies produced in the gastrointestinal tract could prevent the pathogen or toxin from exiting the GI tract to invade the circulatory system or other organs.

BODY:

We had previously described the OmpA proteins in anaerobes and characterized the *ompA* gene and OmpA protein in *B. fragilis* quite extensively. In earlier work, we purified the OmpA proteins from *B. fragilis* and the closely related organism, *Bacteroides distasonis* [7]. Using peptide sequences to construct degenerate primers, we identified the *B. fragilis ompA* gene using reverse genetic techniques (this was prior to the publication of the *B. fragilis* sequence from the Sanger Center). Subsequently, the sequence was confirmed with the data from the Sanger Center, and three additional full length *ompA* homologs were identified both in the ATCC strain, *B. fragilis* 25285, and in the strain more commonly used for molecular manipulations, 638R. The presence of four gene homologs in a single strain suggests that the OmpA protein(s) serves a very important function in the cell.

Construction of an *ompA* deletion mutant. In-frame deletions of *ompA* genes were constructed by a two-step double cross-over technique with the pYT102 suicide vectors [1] (a generous gift from Dr. Michael Malamy). Briefly, approximately 800 bp fragments of the upstream and downstream regions (including approximately 50-100 bp of the beginning and end of the gene to be deleted) of *ompA1* were amplified using specific primers to which appropriate restriction sites were added for subsequent cloning into the suicide vector (Table 1). Oligonucleotide sequences were based on sequence data obtained from the *B. fragilis* NCTC 9343 (ATCC 25285) preliminary genome sequence produced by the Pathogen Sequencing Group at the Sanger Centre (http://www.sanger.ac.uk/Projects/B_fragilis).



Chemically competent *E. coli* DH5α was transformed with plasmid and transformants selected by chloramphenicol. The resultant clone containing the plasmid with the 'up-down'

sequence was mobilized into *B. fragilis* ADB77 using *E. coli* DH5 . and the broad host range mobiliser plasmid pK2317 in a three part mating protocol [1]. The suicide vector pYT102 contains the *B. fragilis thyA* gene and tet^R; recombination at the specific efflux site results in thymine prototrophy (and consequent trimethoprim sensitivity) and tetracycline resistance in the recipient. Cointegrants were selected on plates containing gentamicin (50 µg/ml), rifampicin (50 µg/ml) and tetracycline (2 g/ml, and confirmed by colony PCR using primers designed to detect the recombinant junction. Cointegrant strains were maintained on media with tetracycline.

Cointegrants were subcultured for overnight growth in BHIS broth without antimicrobial selection and then plated on minimal media containing thymine and trimethoprim to select for the second recombination event. Trimethoprim-resistant colonies were screened to confirm that they were tetracycline sensitive, and further screened by PCR with sets of both internal and junction primers to confirm that they were the desired deletion resolution products. Deleted genes were verified by DNA sequencing of the deletion junction and RT-PCR to verify that there was no expression of the gene in question. The sequence of the deletant across the junction is shown in the text box below. The regions upstream and downstream of *ompA* are indicated by green and orange fonts, respectively, two of the primers used are in black italic, and the NCO site created by the junction of the two fragments as described above are indicated in red.

tttttaaaaganttnggatagccgttacgaaaaattttcatgGaacAAaAaTaGgTCAa
agtagctttaggactatatataataaggtgtatcccctgaactttgtagaaagtgcctcg
gaaatttctatttatccaaaacctttagtttcgttggtgttatttaaatagtttcaata
ataaataggaagatttgtaataaccactttaactattaaggtatatgaaaaagattctaa
tgctgctggccttagccggcgttacatctgttgcttctgcg***cagcagactacgattacgg***
ga*ccatgg*tggtggtgtggataaattcggtaaacctatttacctaaaccgagttgtatta
gtagagtctgttaaataagttcggacttgactttctttaaaagaaatcgccctgttatcg
atccggtaacagggcgttctttttttatagataagtaatctgggcttacttatgtatcta

SDS, acid and high salt sensitivity assays. Challenge with SDS, acid, and high salt were performed as described by Wang for *E. coli* OmpA [5] using media and incubation conditions appropriate for *B. fragilis*. Bacteria were grown in BHIS broth to an OD_{600} 0f 0.6 and diluted in either 0.9% NaCl to 10^4 CFU/ml, and plated on BHIS agar containing various concentrations of SDS. Plates were incubated anaerobically for 48 h at 37°C, and CFU were counted. For acid survival, the exponential phase bacteria were diluted 30-fold in PBS. 1/10 volume of the

suspension was mixed with BHIS containing acetic acid to a final pH of 3.8, and incubated at 37°C for 20 minutes. Plates were incubated anaerobically for 48 h at 37°C, and CFU were counted. For the high osmolarity challenge, the 1:30 bacterial suspension was mixed with an equal volume of either 0.9% NaCl or 5M NaCl and incubated at room temperature for 2 h. Plates of varying dilutions were incubated anaerobically for 48 h at 37°C, and CFU were counted.

The increased sensitivity of the *ompA* deletion mutant to both SDS and high salt can be seen in Table 2. Exposure of the parental strain to 5M NaCl for 2 hours resulted in a 3 $\log_{10}$ reduction in growth; the *ompA* deletion mutant did not grow at all after exposure to high salt. Similarly, growth of the parental strain on media containing 0.05-.0.2% SDS resulted in a 3 $\log_{10}$ reduction in growth as compared to growth on media without SDS; the *ompA* deletion mutant did not grow at all on media containing SDS. No change between the parental strain and the *ompA* deletion mutant was seen after exposure to low pH (data not shown.)

Table 2: Effect of high salt and SDS on *Bacteroides fragilis* WAL 108 (parental) and WAL 186 (Δ*ompA1*)

Strain tested	Growth in NaCl		Effect of SDS on growth			
	Growth in 0..9% NaCl	Growth in 5M NaCl	No SDS	0.05% SDS	0.1% SDS	0.2%SDS
WAL 108 (parental)	$1.0 * 10^8$	$5 * 10^5$	$1.0 * 10^8$	$5 * 10^5$	$5.3 * 10^5$	$4.5 * 10^5$
WAL 186 (Δ*ompA1*)	$1.1 * 10^8$	NG	$1.1 * 10^8$	NG	NG	NG

Characterization of the *ompA* deletion mutant grown in media containing added 200 mM NaCl. WAL 108 and WAL 186 were grown overnight in BHIS broth with or without added NaCl (to 200 mM). Gram stain analysis indicated that both WAL 108 and WAL 186 looked similar when grown on normal media (somewhat pleomorphic gram-negative rods). Interestingly, both WAL 108 and WAL 186 had only very small, round forms when grown on the hyperosmolar media (data not shown.)

Quantification of gene expression by quantitative comparative real time RT-PCR

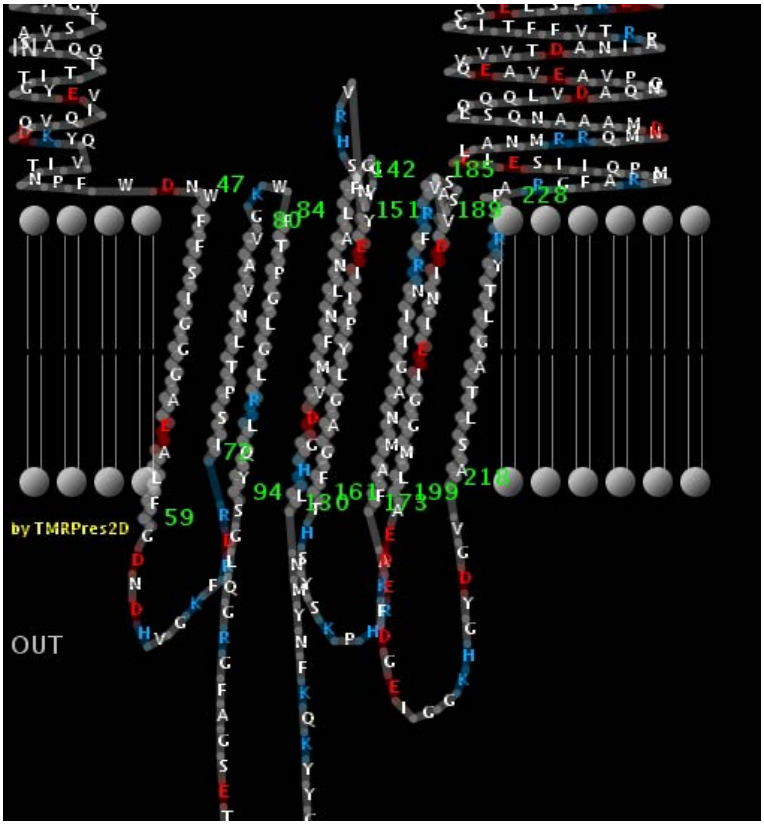
Gene expression was quantified by threshold analysis of SYBR green dye incorporated during the exponential phase of PCR. Briefly, two-step real-time PCR was performed with the Cepheid SmartCycler® using the Quantitect® SYBR® Green one-step RT-PCR kit (Qiagen). RNA expression was normalized to the parental strain by using 16SRNA. Primers were designed to amplify products between 130-170 bp in size (Table 2) and were added to the reactions at a final concentration of 1.0 M each. RNA samples were added to the reactions at a final amount of 200 ng/reaction, except for the 16SRNA samples that were diluted 1:1000, resulting in a final amount of 200 pg/reaction. Expression levels were measured as an amount of cDNA as extrapolated by a cycle threshold (Ct) value from the standard real time PCR growth curve. The Ct was the cycle number at which the growth curve attained exponential growth and was thus the highest concentration of template. In order to rule out any non-specific products resulting from primer dimers, melting curve analysis of the amplified products was performed. Expression results were quantified by the comparative threshold (Ct) approximation method, using the assumption that the PCR growth curve efficiency for all reactions is 100% and that the DNA concentration doubled at each cycle:

	Strain and expression Ct				
gene	**Parental (108)**	***ompA* deletant (186)**	***ompA* disruptant**	**108/ NaCl**	**186/NaCl**
16s rRNA	22.33	23.17	22.07	21.42	21.16
ompA1	20.21	**0**	22.69	**27.62**	0
ompA2	27.76	27.9	27.25	**36.52**	**38.88**
ompA3	25.44	26.74	25.14	**37.21**	**39.54**
ompA4	30.94	**37.59**	31.89	**0**	**0**

A higher Ct value indicates that less product is made. The conclusions from these data are: 1) *ompA1* is the major expressed Omp of the four OmpAs. 2) Other *ompA*s had low level expression that was undetectable on an SDS-PAGE gel; 2) Deletion of *ompA1* also reduces *ompA4* expression; 3) Exposure to 200mM NaCl reduces expression of all four *ompA*s in both the parental and deletant strain; 5) Disruption of *ompA1* still gives product, possibly a truncate and has no effect on *ompA4* expression (this is reasonable in terms of the site of disruption and the location of the primers used in the RT analysis.)

Susceptibility testing. The Spiral Gradient Endpoint (SGE) method, which can differentiate small changes in the minimal inhibitory concentration (MIC) [6,8] was used to measure the antibiotic susceptibilities of the strains. All test agents were prepared according to the manufacturers' instructions. Selected stock concentrations of antimicrobial agents were deposited onto Brucella blood agar plates in a spiral pattern using the spiral plater (Autoplate 4000, Advanced Instruments; Norwood, MA) which resulted in a radially decreasing concentration gradient from the center to the edge of the plate. Bacterial strains were resuspended in Brucella broth at a standard McFarland concentration of 0.5 and deposited on the plates. The plates were incubated for 24-48 hours at 37^{o}C under anaerobic conditions. The distance of growth from the edge toward the point of no growth was measured and the SGE computer software used to convert growth radii to MIC values (μg/ml). Susceptibility studies were performed on at least three independent occasions. Since there are no established resistance breakpoint values in anaerobes for the tested antibiotics, a ≥2-fold difference in susceptibility was considered significant. MICs were expressed as the next two-fold dilution for convenient comparison, although more accurate determinations are made by the spiral plater, and the degree of accuracy is $\pm$0.26 of a twofold dilution [8] (compared to the $\pm$ one two-fold dilution accuracy of the standard NCCLS agar dilution method.). Susceptibility testing was performed for a wide variety of antimicrobials including β-lactams (ampicillin, cefoperazone, cefoxitin, cephalexin, ceftizoxime), carbapenems (doripenem, ertapenem, faropenem, imipenem, meropenem), quinolones (ciprofloxacin, gatifloxacin, norfloxacin, levofloxacin, moxifloxacin), chloramphenicol, metronidazole, clindamycin, erythromycin and tetracycline. No significant change was seen in MICs of a variety of antimicrobials for the deletion mutant compared to the parental strain.

Structural predictions for OmpA. In order to determine, as a first estimate, where to place the FLAG TAGS for eventual placement of the vaccine antigen epitopes, we analyzed the predicted secondary structure of OmpA with a variety of bioinformatic analysis programs, and used a combination of the results to formulate a model (shown below). Based on this model, we will also conduct analysis on the loop portions to predict coil and helix region, and determine where to place the first experimental tags.



Comparison of parental and *ompA* deletant strains in invasion assays of brain microvascular endothelial cells (BMEC) and macrophages. This assay has thus far only been done once (in the laboratory of Dr. Prasadarao Nemani (University of Southern California) [2,3]. Briefly, bacteria were added to confluent monolayers of BMEC and incubated for ~1.5 hours at 37 ^{o}C. The monolayers were washed and the number of cell associated bacteria determined after the BMEC were lysed with 0.5% Triton x. The number of intracellular bacteria was determined after extracellular bacteria were eliminated by incubation of the monolayer with antibiotic. The released intracellular bacteria were plated on blood agar and then counted. Additionally, the entry and intracellular expression of *B. fragilis* in macrophages was measured [4]. In these initial studies, we determined that *B. fragilis* could survive the incubation in the CO_2 atmosphere used for these studies, but we did not observe any binding or invasion of the bacteria. There are several possible explanations, and we will be repeating the experiment under different conditions. For example, we will conduct the assay in a totally anaerobic environment, to allow the *B. fragilis* to be metabolically active. We will also test several alternate strains of *B. fragilis*, as well as conducting the experiment with a mixture of *E. coli* and *B. fragilis* (appropriate since *B. fragilis*, like other anaerobes, is generally part of a mixed infection.)

Table 1: Strains, plasmids and primers used in this study

Strains		**Description or relevant marker**	**Source or reference**
	WAL 3501	ATCC 25285, type strain	ATCC
	WAL 26	*B. fragilis* 638R	Dr. C. J. Smith
	WAL 67	*B. fragilis* 638R/pFD516::'*ompa*'; erythromycin^{R}	This study
	WAL 108	*B. fragilis* ADB77; TM400 ΔthyA, rifampicin^{R}, trimethoprim^{R}	Baughn and Malamy (2001)
	WAL 174	*B. fragilis* ADB77/pYT102::ompAupdown; tetracycline^{R}	This study
	WAL 186	*B. fragilis* ADB77 ΔompA	This study
		E. coli/pRK	
		E. coli DH5α	Dr. C. J. Smith
		E. coli DH5α/pFD516::'*ompa*'; erythromycin^{R}	This study
		E. coli DH5α/pYT102::ompAupdown; tetracycline^{R}	This study
Plasmids			
	pFD516	erythromycin^{R}	Dr. C. J. Smith
	c	erythromycin^{R}	This study
	pYT102	tetracycline^{R}	Baughn and Malamy (2001)

	pYT102::ompAupdown	tetracycline^{R}	This study
Primers for RT PCR analaysis of ompAs1-4			
	Primer	**Sequence (5' to 3')**	
	OmpA1-F	CAGCTGATTTTGTGAAGAGTGG	
	OmpA1-R	CCCGGGAAACGATAAGTCAAA	
	OmpA2-F	CAACCCCGACAACTTTGATT	
	OmpA2-R	TAGGCCTTGGCAAACGTAAG	
	OmpA3-F	TTTCATGCCGACACTTTCTG	
	OmpA3-R	ACGGTAACGTGCCTGGATAC	
	OmpA4-F	GGACAGCCTGCCGATACTTA	
	OmpA4-R	TTACCAAACGGAGACGGAGA	
Primers used in real time RT PCR analysis			
	OmpA1-F	GGA TAT GAC GGT GTT GCC AG	
	OmpA1-R	TAG CAG CAG CCA TGT CAT TC	
	OmpA2-F	TAG AAG GTG CAT GGA CTA CT	
	OmpA2-R	AAC CGC CAA TAG CAT TGG AC	
	OmpA3-F	ACT CCG CTG ATC AAT GTG TC	
	OmpA3-R	CGT CTG CAC GCA TAG TGA AG	
	OmpA4-F	CCA AGA TCG ACG ACT ATG CT	
	OmpA4-R	TTC TGG TTC CAC TTG GCA CT	

KEY RESEARCH ACCOMPLISHMENTS:

- **Construction of *B. fragilis* lacking the *ompA* gene (*ompA* deletant) and partial characterization of this strain**
- **Structural analysis of *B. fragilis* OmpA to determine where FLAG tag epitopes should be placed**
- **Initial studies of regulation of *ompA* transcription using RT-PCR and morphologic characterization of strains**
- **Provided collaborators Drs. Prasadarao, Nitzan and Onderdonk with parental and *ompA* deletant strains to begin virulence, animal model, and cytokine assay studies.**
- **Initial virulence assays with brain microvascular endothelial cells (BMEC) and macrophages were carried out.**

REPORTABLE OUTCOMES:

H.M. Wexler, 2005. The function of the OmpA outer membrane protein in *Bacteroides fragilis*. Abstracts. American Society for Microbiology General Meeting, Atlanta, GA.

H.M.Wexler, L. Pumbwe, E.K. Read, and T.J. Tomzynski. 2006. Function of the OmpA outer membrane protein in *Bacteroides fragilis.* Submitted for publication.

H.M. Wexler. 2006. Abstract submitted for the DoD MHRF, May 1-4.

CONCLUSION:

OmpA is one of the most abundant outer membrane proteins in *Bacteroides fragilis*, and all four homologs of the *ompA* gene are transcribed, although only OmpA1 is detectable on SDS-PAGE and on Western blot analysis with anti-OmpA antisera. OmpA is important in maintaining cell structure: 1) the OmpA disruption mutant is shorter and rounder than the parental strain; 2) the OmpA deletant is more sensitive to SDS and to high salt concentrations than the parent strain; 3) the bacterium responds to high salt stress by shutting down OmpA production and assuming a tight, small, round morphology. We were able to obtain a deletion mutant of OmpA using a two-step recombination technique, and will use this same technique to insert the modified OmpA back into the deletant strain. Since the strains will not have any OmpA unless we succeed in inserting it,

successful matings will be fairly simple to identify. Based on data that we will collect on the virulence and immunological assays, we will further determine which loops should be modified for eventual use in a vaccine vector.

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APPENDICES: All figures and tables are embedded in the text.

SUPPORTING DATA: All figures and tables are embedded in the text.